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Plant-fungal association in trees: Insights into changes in ecological strategies of *Peroneutypa scoparia* (*Diatrypaceae*)

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ABSTRACT

Fungal endophytes comprise a highly diverse group of particular interest for their relevant implications to the ecosystems they inhabit. The objective of this study was to infer the phylogenetic affinity between strains of *Peroneutypa scoparia* exhibiting different lifestyles to elucidate possible shifts in ecological roles. Specimens and living cultures used in the present study were obtained from decaying wood and from live stem tissues of the invasive host species *Broussonetia papyrifera*. The similarity between the fungal strains was studied through molecular analyses. The results showed a close phylogenetic link and high genetic similarity between endophytic and saprotrophic strains. The main findings suggest that *P. scoparia* has primary access to the substrate as an endophyte and then this organism may change its use of the available resources presenting a saprotrophic growth. These results provide valuable information about the roles that diatrypaceous fungi play as endophytes or as decaying wood inhabitants and contributes to evaluate the ecological significance of this group.

Introduction

Fungal endophytes, *i.e.* fungi that live within plant tissues without causing symptoms, are found in all plant lineages (Arnold, 2008; Petrini, 1991; Rodriguez et al., 2009). This diverse group of fungi can modulate the ecology of plant communities, conferring resistance to abiotic and biotic stresses (Rodriguez et al., 2009).

The ecological roles of endophytes are just starting to be elucidated. So far, it is known that endophytes may be neutral, parasitic or mutualistic inhabitants of their hosts (Arnold, 2008; Hyde and Soytong, 2008, Partida-Martínez and Heil, 2011). The high diversity of endophytes harbored by a single host probably includes species with the ability to either play just one of these roles or change roles over time or under certain conditions (Arnold, 2008). Previous studies based on molecular and enzymatic approaches have suggested that fungal organisms that occur as endophytes can switch their nutritional mode (from saprotrophic to parasitic or vice versa) or/and lifestyles (soil, fungi and decaying and living plants) (Chaverri and Samuels, 2013; Delaye et al., 2013; Duong et al., 2008; Hyde et al., 2007; Oses et al., 2008; Promputtha et al., 2007; Promputtha et al., 2010; Parfitt et al., 2010).

Many studies have assessed the connection between parasitic and saprotrophic fungi in wood; however, few of them performed an experimental design to establish a direct relationship between these nutritional modes or habitat preferences (Álvarez-Loayza et al., 2011; Chaverri and Samuels, 2013).

The diversity of members of the family *Diatrypaceae* (Xylariales, Ascomycota) have been extensively studied in wood of different hosts (Rappaz, 1987; Vasilyeva and Stephenson, 2004, among others). Species of this family have been mostly described as saprotrophic on dead wood and only few species have been characterized as pathogens (Trouillas and Gubler, 2010). Previous reports indicate that members of this group are able to grow in living trees as endophytes; *Libertella* sp. on *Picea excelsa* (Carroll et al., 1977), *Cryptosphaeria populina* (Chapela, 1989) and *Cryptosphaeria lignyota* on *Populuts tremuloides* Michx (Hutchinson, 1999) and more recently *Peroneutypa scoparia* was recorded from *Garcinia* species (Phongpaichit et al., 2007).

In Argentina several works have been carried out in order to characterize the diversity of saprotrophic species of *Diatrypaceae* (Carmarán and Romero, 1992; Carmarán, 2002; Carmarán et al., 2006; Pildain et al., 2005). In a previous work, we isolated endophytic strains of *P. scoparia* (anamorphic state, *Libertella* sp.) from living branches of *Broussonetia papyrifera* (L.) L'Her. ex Vent,

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considered an invasive tree (de Errasti et al., 2010), turning this tree species into an interesting model to study the relationship between strains that occur as endophytes and as fallen wood-inhabitants of the same species of fungi.

The aims of the present study were to determine the occurrence of an endophytic stage of saprotrophic fungi within *Diatrypaceae*, by performing an ecological survey at the field, and to evaluate the relationship between the obtained strains through phylogenetic analyses based on DNA sequence comparison of the ITS region and the β-tubulin gene.

Materials and methods

Study area and plant material

The study area is located in Dr. Carlos Spegazzini Reserve—a natural Mycological Reserve within an urban area in Lomas de Zamora district (34° 47′ S; 56° 56′ W), Buenos Aires province, Argentina. This area is characterized by a slightly acid soil, with pH values ranging from 5.4 to 5.8. The maximum and minimum average five-year temperatures are 22.2 °C and 10.7 °C, respectively. The average rainfall is 938 mm, average relatively humidity is 73%. The land occupies approximately 60 ha and the plant community is characterized by a naturalized forest (De Magistris, 1996).

Broussonetia papyrifera (L.) L'herit (Moraceae), the host tree from which we have previously isolated diatrypaceous fungi as woody-tissue endophytes (de Errasti et al., 2010), was chosen to carry out the present study. In Argentina, *B. papyrifera* is an exotic invasive tree (INBIAR, 2013) in protected areas due to its high colonization rate.

Isolates from living trees

The diatrypaceous strains used in the present study were those previously obtained from woody tissue of living trees of *B. papyrifera* (de Errasti et al., 2010). They are deposited in the culture collection as *BAFCcult* 3321, 3322, 3390 and 3391 (Culture collection of the Facultad de Ciencias Exactas y Naturales, University of Buenos Aires).

Experimental design

To evaluate the occurrence of an endophytic stage of saprotrophic strains in the same host, we carried out the following analyses on the same individual trees from which we have previously isolated diatrypaceous fungi as endophytic strains. A total of five trees of *B. papyrifera* were included in this study and three healthy branches were cut off from each tree (2 – 6 cm in diameter), resulting in a sampling of 15 branches (modified from Hendry et al., 2002). From each branch, two 10 cm-long sections (herein after referred to as "units") were cut, kept in sterile polyethylene bags and processed in the laboratory within 48 h after collection (according to the Section "Survey of newly saprotrophic infections of diatrypaceous fungi"). The remaining parts of the branches (remaining branches) were labeled and left on the ground to perform further analyses (Fig. 1).

Survey of preceding saprotrophic infections of diatrypaceous fungi

To examine infections established previous to the beginning of this study, fallen branches with typical diatrypaceous stromata were collected from the sampling area. The sampling was performed at the beginning of the study and six months later. At the laboratory, isolates were obtained from the hamathecium.

Survey of newly saprotrophic infections of diatrypaceous fungi

To study the development of new saprotrophic infections two procedures were carried out. First, the cut units, kept in sterile polyethylene bags, were sterilized by autoclave and then within 48 h, they were returned to the sampling site (herein referred to as "sterilized units") to the same location point of the "remaining branches". From remaining branches, units 10 cm-long were cut (herein referred to as "field units") and left on the ground near to sterilized units. Then, the sterilized units and the field units were checked to detect the emergence of diatrypaceous fungi (teleomorphic or anamorphic stromata) six months and one year after the sampling. When specific fungal structures were noticed, isolates from the hamathecium were performed.

The experiment began in April 2007 (autumn), when the healthy branches were cut off from the trees, and continued until the same month the following year. Therefore, the two types of units (sterilized *vs* field) were subjected to the same environmental conditions.

Evaluation of epiphytic and endophytic fungi of bark

To discard potential colonization of the branches by epiphytic fungi, portions of bark from each individual tree were analyzed by (a) pressing the external surface of the bark on a sterile plate with malt extract agar (MEA 2%) and (b) plating small pieces of bark, previously surface-sterilized, on the same medium. The presence of diatrypaceous fungi was assessed for the following 15 days.

Fungal identification

Two replicates of each strain, obtained from the different procedures, were grown on MEA and potato dextrose agar (PDA) for at least 10 days. Pure cultures were examined periodically for sporulation. The morphological identification of the strains was carried out only for members of the family *Diatrypaceae*. Identified species were preserved in the *BAFCcult* (Holmgren et al., 1990).

DNA extraction, amplification and sequencing

The diatrypaceous strains obtained from the different procedures (BAFCcult 3320, BAFCcult 3323, BAFCcult 3324, BAFCcult 3325, BAFCcult 3326, BAFCcult 3327, BAFCcult 3328) and two diatrypaceous fungi obtained in our previous work (BAFC 3321, BAFC 3322) (de Errasti et al., 2010) were grown on MEA 0.1% (w/v) and incubated at 25 °C for 21 days in light/darkness. DNA was extracted from the cells using the UltraCleanTM Microbial DNA Isolation Kit (MO BIO Laboratories Inc., Solana Beach, USA) according to the manufacturer's instructions. The ITS region of the strains was amplified using the universal primers ITS1 and ITS4 (White et al., 1990), whereas a fragment of the β-tubulin gene was amplified using the primers Bt2b and T2 (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997). In some cases, best amplification results were achieved by adding 6% bovine serum albumin (BSA, Promega Corp.) to the PCR reaction mix. PCR products were purified using a QIAGEN Gel Extraction kit (QIAGEN Inc.). Both strands of each fragment were sequenced by Macrogen Service Center. *Xylaria berteri* and *X. curta* were chosen as outgroups.

Molecular and phylogenetic analyses

A total of 34 sequences from ITS and 31 from β-tubulin genes, including 10 from isolated strains from the Dr. Carlos Spegazzini reserve (de Errasti et al., 2010), were used in the molecular analyses (Table 1). Environmental samples of NCBI BLAST were tested to identify sequences with high similarity. No significant results were obtained. The sequences obtained in this study were pair-wise compared to estimate percentage of similarity.

A dynamic homology analysis was performed using the program POY 4 (Varón et al., 2010). The commands build (10) and swap (100) were used (cost 5 was assigned for gap opening). To determine the